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Methods 130 (2017) 51–62

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Hypoxia and hypoxia response-associated molecular markers in esophageal cancer: A systematic review

Jurgen Peerlings [a,b,1,*], Lien Van De Voorde [a,1], Cristina Mitea [b], Ruben Larue [a], Ala Yaromina [a], Sebastian Sandeleanu [a], Linda Spiegelberg [a], Ludwig Dubois [a], Philippe Lambin [a], Felix M. Mottaghy [b,c]

[a] MAASTRO Clinic, Department of Radiation Oncology, GROW – School for Oncology and Developmental Biology, Maastricht University Medical Centre+, Maastricht, The Netherlands
[b] Department of Radiology and Nuclear Medicine, Maastricht University Medical Centre+, Maastricht, The Netherlands
[c] Department of Nuclear Medicine, University Hospital RWTH Aachen University, Aachen, Germany

ARTICLE INFO

Article history:
Received 1 May 2017
Received in revised form 8 June 2017
Accepted 4 July 2017
Available online 10 July 2017

Keywords:
Hypoxia
Esophagus
Oncology
Treatment outcome
Survival

ABSTRACT

Purpose: In this systematic review, the existing evidence of available hypoxia-associated molecular response biomarkers in esophageal cancer (EC) patients is summarized and set into the context of the role of hypoxia in the prediction of esophageal cancer, treatment response and treatment outcome.
Methods: A systematic literature search was performed in Web of Science, MEDLINE, and PubMed databases using the keywords: hypoxia, esophagus, cancer, treatment outcome and treatment response. Eligible publications were independently evaluated by two reviewers. In total, 22 out of 419 records were included for systematic review. The described search strategy was applied weekly, with the last update being performed on April 3rd, 2017.
Results: In esophageal cancer, several (non-)invasive biomarkers for hypoxia could be identified. Independent prognostic factors for treatment response include HIF-1α, CA IX, GLUT-1 overexpression and elevated uptake of the PET-tracer ^{18}F-fluoroerythronitroimidazole (^{18}F-FETNIM). Hypoxia-associated molecular responses represents a clinically relevant phenomenon in esophageal cancer and detection of elevated levels of hypoxia-associated biomarkers and tends to be associated with poor treatment outcome (i.e., overall survival, disease-free survival, complete response and local control).
Conclusion: Evaluation of tumor micro-environmental conditions, such as intratumoral hypoxia, is important to predict treatment outcome and efficacy. Promising non-invasive imaging-techniques have been suggested to assess tumor hypoxia and hypoxia-associated molecular responses. However, extensive validation in EC is lacking. Hypoxia-associated markers that are independent prognostic factors could potentially provide targets for novel treatment strategies to improve treatment outcome. For personalized hypoxia-guided treatment, safe and reliable makers for tumor hypoxia are needed to select suitable patients.

Contents

Abbreviations: AC, adenocarcinoma; ARCON, accelerated radiotherapy with carbogen breathing and nicotinamide; CA IX, carbonic anhydrase; CCRT, concurrent chemoradiotherapy; CR, complete response; Cu-ATSM,, copper-62 labeled diacetyl-bis (N4-methylthiosemicarbazone); DFS, disease-free survival; EC, esophageal cancer; ESCC, esophageal squamous cell carcinomas; 18F-FAZA,, 18F-fluoroazomycin arabinoside; 18F-FETA,, [18F]fluoroetanidazole; 18F-FETNIM, 18F-fluoroerythronitroimidazole; 18F-FMISO, 18F-fluoromisonidazole; 18F-HX4, 18F-3-fluoro-2-(4-((2-nitro-1H-imidazol-1-yl)methyl)-1H-1,2,3-triazol-1-yl)propan-1-ol; GLUT-1, glucose-transporter-1; HAP, hypoxia-activated prodrug; HIF, hypoxia-inducible factor; HRE, hypoxia response element; LC, local control; MESH, Medical Subject Headings; MRI, magnetic resonance imaging; OE-MRI, oxygen-enhanced magnetic resonance imaging; OS, overall survival; PET, positron-emission tomography; PDT, photodynamic therapy; ROS, reactive oxygen species; SUV, standard uptake values; VEGF, vascular endothelial growth factor.

* Corresponding author at: MAASTRO Clinic, Dr. Tanslaan 12, 6229 ET Maastricht, P.O. 3035, Netherlands.
E-mail address: Jurgen.peerlings@maastro.nl (J. Peerlings).
[1] Both authors contributed equally.

http://dx.doi.org/10.1016/j.ymeth.2017.07.002
1046-2023/

1. Introduction

Hypoxia is one of the hallmarks of cancer and has been associated with a more aggressive tumor phenotype, a higher likelihood of metastatic progression and resistance to (chemo)radiotherapy [1]. Hypoxia occurs when tissue oxygen demand (e.g., increased metabolism) exceeds oxygen supply (e.g., acute and/or chronic vascular changes, anemia, malfunctioning hemoglobin). In normal tissue, acute hypoxia (i.e., perfusion-limited) is resolved by physiological homeostasis while in cancerous tissue, additional chronic hypoxia (i.e., diffusion-limited) is more likely to manifest. The rapid and uncontrollable tumor growth requires large amounts of nutrients and therefore triggers neo-angiogenesis. However, the resulting tumor neo-vasculature is highly chaotic and inefficient. Oxygenation of tumor regions surrounding perfused blood vessels therefore depends on a diffusion-gradient, relative to the intravascular oxygen partial pressure (pO_2). Generally, the diffusion-gradient is limited to 100–180 μm, thus inducing chronic hypoxia in remote regions [1].

Clinically, hypoxia is thought to be a key factor contributing to treatment resistance and poor patient prognosis [2]. Although neoadjuvant therapy (i.e., CROSS regimen with weekly carboplatin (2 mg/ml/min AUC) and paclitaxel (50 mg/m^2) for 5 weeks, concurrent radiotherapy (41.4 Gy in 23 fractions, 5 days per week), followed by surgery) has been proven to be valuable in esophageal cancer (EC), prognosis remains dismal with approximately 20% complete responders (5 yr overall survival = 20–30% [3,4]), making EC the sixth most lethal cancer type in 2012, worldwide [5]. In 2016, over 15.000 patients died from EC in the USA alone [5]. Most EC contain hypoxic areas with a higher percentage in the adenocarcinomas, potentially explaining the poor treatment outcome for these patients [6]. About half of the patients treated with definitive chemoradiation will suffer from a locoregional recurrence. For effective radiation treatment, the presence of molecular oxygen is essential. Under normoxic conditions, ionizing radiation leads to the formation of free radicals and reactive oxygen species (ROS), which can damage DNA. Free radicals produced in the critical target can be fixed in the presence of oxygen, leading to irreversible DNA damage. In hypoxic conditions however, free radicals are reduced and hypoxic regions becomes 2–3 times more radio-resistant, which may explain low rates of complete response (CR) and local control (LC) [1,7]. Accordingly, patients with hypoxic EC might need a different, personalized treatment approach to reach therapeutic success.

Since tumor hypoxia cannot be predicted based on clinical size, stage, or grade, there is a need for molecular biomarkers that can assess hypoxic status in EC. Such biomarkers could be used to detect hypoxic tumor status at an early stage, evaluate treatment response, predict prognosis in EC patients and select patients for suitable, personalized treatment options.

In this systematic review, we provide an overview of hypoxia response-associated biomarkers in EC patients and aim to evaluate the prognostic value of elevated expression rate of hypoxia-associated biomarkers with regard to treatment outcome and efficacy [i.e., overall survival (OS), disease-free survival (DFS), CR, and LC]. Markers that are independent prognostic factors could potentially provide targets for novel treatment strategies. In addition, several known methods to improve treatment outcome will be discussed in relationship to these hypoxia-associated biomarkers.

2. Material and methods

2.1. Systematic search strategy

The research question for this systematic review was defined as: "What are the known hypoxia-associated molecular markers in patients with EC and how does elevated expression associate with treatment outcome and response?".

To consider the research question, a comprehensive PRISMA-based literature search was performed to identify relevant studies published in PubMed (National Center for Biotechnology Information, NCBI), MEDLINE (U.S. National Library of Medicine, using NCBI), or Web of Science (Thomson Reuters). The electronic databases were explored using a PICOS-based search string containing a free-text or Medical Subject Headings (MeSH) construction of 5 key search terms: 'hypoxia' AND 'esophagus' AND 'cancer' AND ('treatment outcome' OR 'treatment efficacy'). For each search term, all known synonyms and associated keywords were included in the search string using Boolean OR-operators. A detailed description of the entire search strings can be found in Appendix A1 [8]. The complete search strategy was applied weekly, with the last update being performed on April 3rd, 2017.

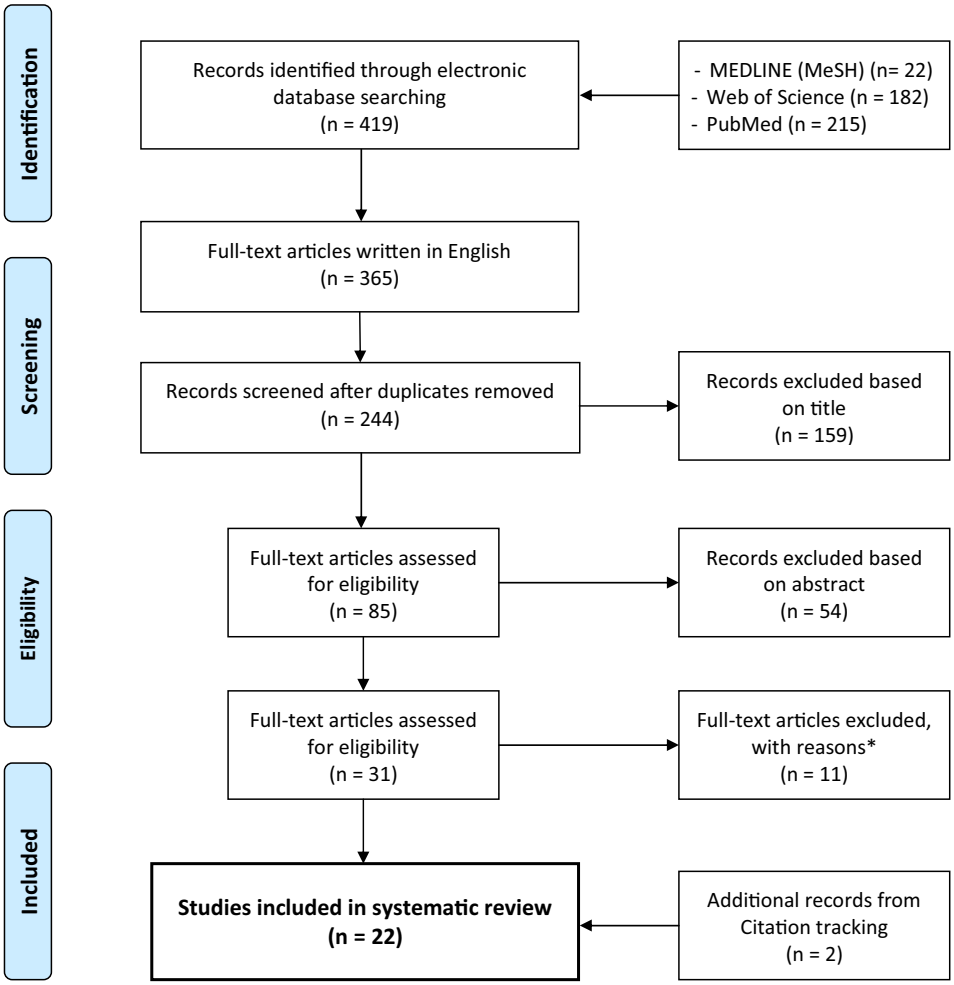


Fig. 1. Flowchart of information through different phases of systematic review. *Reasons for exclusion on full-text level were: not fitting PICOS-based research question (n = 6), use of preclinical tumor models (n = 4), or unsuitable to extract survival data (n = 1).

2.2. Study selection

Articles were eligible for inclusion when corresponding to the predetermined eligibility criteria: (1) the patient population consisted of human adults diagnosed with esophageal cancer or clinically acquired EC tissue samples; (2) the index tests were all tests able to assess tumor hypoxia; (3) treatment outcome had to be evaluated and correlated with hypoxia. Only full-text articles written in English were retrieved from the electronic databases. If full-text content was not available to us, the corresponding author was contacted to retrieve the printed publication. Next, duplicate findings were manually discarded to ensure that no data overlap occurred. Further selection was performed by applying several exclusion criteria: (1) reviews, letters, abstracts, case studies, etc.; (2) studies using only esophageal cell lines or animal-based tumor models; (3) studies aiming to investigate the molecular mechanisms of hypoxia; (4) studies that did not correlate expression rate of hypoxia-associated markers with treatment outcome or efficacy (i.e., CR, LC, OS, or DFS). Additional eligible articles were retrieved by manually cross checking reference lists of relevant articles and reviews (citation tracking). Furthermore, databases were searched to retrieve studies exploring additional methods for non-invasive hypoxia-assessment in cancer patients by performing a secondary search including the MeSH-terms 'Hypoxia' AND ('MRI' OR 'SPECT' OR 'PET' OR 'CT'). This search was not specific for esophageal cancer and will be reviewed in the second part of this manuscript.

2.3. Data extraction

Two investigators (J.P. and L.VDV.) performed each step of this protocol independently (i.e., systematic search, defining eligibility, and data extraction). In cases of disagreement and consensus could not be reached, a third party (L.D.) was consulted to adjudicate. From the included articles, data was extracted concerning study characteristics (i.e., author, publication year), patient characteristics (i.e., number of subjects, country of origin, specimen type, tumor cell type), measurement characteristics (i.e., method of quantifying hypoxia, marker type, definition of hypoxia, percentage of hypoxic elements), and treatment strategy and response outcome characteristics (i.e., OS, DFS, CR, and LC). CR is defined as the total disappearance of a tumor, and LC as the arrest of cancer growth at the site of origin (i.e., stable tumor volume). Survival is assessed by OS, defined as the time interval from end of primary therapy until last known survival data or death, and DSF that is defined as the time interval after primary treatment and the first signs of recurrence, metastasis, or cancer-related disease. Furthermore, statistical outcome was extracted with p-values defining the prognostic/predictive power. P-values <0.05 indicated statistically significant differences in treatment outcome between high and

Table 1
Extracted data concerning Overall survival (OS) and Disease-free survival (DFS) in esophageal squamous cell carcinoma (SCC) and adenocarcinoma (AC). IHC = immunohistochemistry. (*) P-values <0.05 indicate significant differences.

Ref	Author	publication year	Country	Specimen	Index test	Marker	hypoxia criteria (threshold expression rate/definition of hypoxia	Tumor type	# samples	Hypoxic sample (%)	OS				DFS				Independent Prognostic factor
											hypoxic vs. non-hypoxic sample	P-value* Kaplan-Meier model	Cox proportional hazard model		hypoxic vs. non-hypoxic sample	P value* Kaplan-Meier model	Cox proportional hazard model		
													Univariate P-value*	Multivariate P-value*			Univariate P-value*	Multivariate P-value*	
[9]	Birner et al.	2011	Austria	Surgical	IHC	CAIX	>median staining intensity* staining rate	All	330	44.5%	30% vs. 54%	0.001	<0.001	0.009	24% vs.52%	0.001	<0.001	0.001	Yes
								AC	182	46.7%	37% vs.59%	0.001	0.007	0.027	33% vs. 55%	0.001	0.007	0.019	Yes
								SCC	148	41.9%	19% vs.48%	0.001	0.001	0.043	12% vs. 45%	0.001	<0.001	0.01	Yes
[10]	Chen et al.	2009	China	Surgical	IHC	Beclin-2	<10% cytoplasm/cytomembrane staining	SCC	54	33.3%	–	0.004	–	–	–	–	–	–	–
						HIF-1 α	>mean cytotoplasmic expression	SCC	54	46.3%	–	0.052	–	–	–	–	–	–	–
						Beclin-2 and HIF-1α	Low Beclin-2 and High HIF-1α	SCC	54	24.1%	–	0.001	–	–	–	–	–	–	–
[11]	Chiba et al.	2010	Japan	Biopsy	IHC	GLUT-1	>30% membrane expression	SCC	25	28.1%	–	–	–	–	28.5% vs. 30.1%	0.0405	0.0405	0.016	yes
[12]	Driessen et al.	2006	The Netherlands/ Belgium	Surgical	IHC	CAIX	>median membranous staining intensity	AC	39	49.0%	27% vs. 47%	0.008	–	0.017	–	–	–	0.041	yes
						VEGF	>median cytoplasmic staining intensity	AC	39	69.2%	–	–	>0.05	>0.05	–	–	>0.05	>0.06	no
[13]	Jomrich et al.	2014	Austria	Surgical	IHC	CAIX	≥10% stromal staining	Total	361	11.6%	28% vs. 44%	0.013	–	0.196	25% vs. 37%	0.007	–	0.081	no
								AC	206	11.2%	42% vs. 47%	0.142	–	0.256	40% vs. 41%	0.121	–	0.055	no
								SCC	155	12.3%	10% vs. 39%	0.045	–	0.933	8% vs. 33%	0.029	–	0.807	no
[14]	Katsuta et al.	2005	Japan	Surgical	IHC	HIF-1 α	>10% nuclear or distinct cytoplasmic staining	SCC	48	70.8%	70.6% vs. 92.9%	0.3408	–	–	20.6% vs. 0%	0.09	–	–	–
						VEGF	>30% cytoplasmic staining	SCC	48	60.4%	65.5% vs. 89.5%	0.4659	–	–	26.1% vs. 5.6%	0.22	–	–	–
[15]	Kimura et al.	2004	Japan	Surgical	IHC	HIF-1 α	>mean nuclear/cytoplasmic expression	SCC	47	36.1%	–	0.044	–	–	–	–	–	–	–
						VEGF	>10% cytoplasmic staining	SCC	47	53.2%	–	0.0012	–	–	–	–	–	–	–
[16]	Koukourakis et al.	2001	Greece	Biopsy	IHC	HIF-1 α	nuclear staining	SCC	37	51.0%	–	0.08	–	–	–	0.14	–	–	–
						HIF-2 α	nuclear staining	SCC	37	13.5%	–	0.03	–	–	–	0.1	–	–	–
[17]	Kurokawa et al.	2003	Japan	Surgical	IHC	HIF-1 α	>10% nuclear or distinct cytoplasmic staining	SCC	130	30.8%	40% vs. 58%	0.0007	0.0011	0.1669	–	–	–	–	no
[18]	Ling et al.	2006	Germany	Biopsy + Surgical	IHC	HIF-1 α protein	nuclear staining	Total	53	33.3%	–	–	0.53	–	–	–	–	–	no
					RNA assay	HIF-1 α mRNA	Ratio tumor-to-normal epithelium >1	Total	53	53.8%	–	–	0.78	–	–	–	–	–	no
								AC	22	15.4%	–	–	0.59	–	–	–	–	–	no
								SCC	31	38.5%	–	–	0.23	–	–	–	–	–	no
[19]	Matsuyama et al.	2004	Japan	Surgical	IHC	HIF-1 α	>mean nuclear/cytoplasmic expression	SCC	215	95.0%	43% vs. 52%	0.109	0.112	–	47% vs. 55%	0.024	0.027	0.142	no
[20]	Munipalle et al.	2011	United Kingdom	Biopsy	IHC	HIF-1 α	≥10% nuclear staining	SCC	36	52.8%	26% vs. 18%	0.908	–	<0.001	–	–	–	–	yes
[21]	Ogane et al.	2010	Japan	Surgical	IHC	HIF-1 α	nuclear staining	SCC	96	67.7%	–	0.034	0.0322	0.227	–	0.019	–	–	yes
[22]	Ogawa et al.	2011	Japan	Biopsy	IHC	HIF-1 α	>10% staining	SCC	25	18.2%	–	>0.05	–	–	18.2% vs. 39.8%	0.0009	0.0009	0.001	yes
[23]	Schreurs et al.	2014	The Netherlands	Patient	PET scan	^{18}F-FDG	SUVmax (FDG) > 3.67	Mix	47	–	23.3% vs.56.6%	0.301	–	–	17% vs. 62%	0.022	–	–	–
[24]	Sohda et al.	2004	Japan	Biopsy	IHC	HIF-1 α	>10% cell staining	Mix	65	58.5%	–	0.472	–	–	–	–	–	–	–
[25]	Takala et al.	2011	Finland	Surgical	IHC	HIF-1 α	>5% nuclear staining	Mix	80	71.3%	–	0.223	–	–	–	–	–	–	–
						VEGF	>25% cytoplasmatic staining	Mix	84	71.4%	–	0.84	–	–	–	–	–	–	–
[26]	Tanaka et al.	2008	Japan	Surgical	IHC	CAIX	>median staining rate and intensity	SCC	127	59.8%	33.3% vs. 66.5%	0.0003	0.0006	0.7859	–	–	–	–	no
[27]	Tzao et al.	2008	Taiwan	Surgical	IHC	HIF-1 α	>10% positive staining	SCC	85	61.2%	–	0.002	–	0.044	–	–	–	–	yes
						VEGF	>10% positive staining	SCC	85	65.9%	–	0.001	–	0.035	–	–	–	–	yes
[28]	Winther et al.	2013	Denmark	Biopsy	Gene Clustering	15 hypoxia-assoc. genes	weighted hypoxia gene expression signature	Mix	56	31.0%	12.5% vs. 48.6%	0.07	>0.05	0.11	12.5% vs. 48.6%	0.13	0.02	0.22	no
[30]	Zhang et al.	2014	China	Surgical	IHC	HIF-1 α	>median staining intensity * staining rate	SCC	136	52.2%	25.3% vs. 36.9%	0.031	–	0.22	22.5% vs. 35.4%	0.042	–	0.35	no
								Locoregional SCC	67	41.8%	32.1% vs. 51.3%	0.044	–	0.039	32.1% vs. 51.3%	0.049	–	0.051	yes
								Metastatic ESSC	69	62.3%	11.5% vs. 16.3%	0.847	–	–	–	0.563	–	–	no

Table 2
Extracted data concerning complete response (CR) and Local control (LC) in esophageal squamous cell carcinoma (SCC) and adenocarcinoma (AC). CCRT = concurrent chemoradiotherapy, PDT = photodynamic therapy. (*) P-values <0.05 indicate significant differences.

Ref	Author	Publication year	Country	Specimen	Index test	Marker	hypoxia criteria (threshold expression rate)	Tumor type	# Sample	Hypoxic sample (%)	Therapy	CR (5 yr)		LC (5 yr)			Independent Predictive factor
												hypoxic vs. non-hypoxic group	CR p-value*	hypoxic vs. non-hypoxic group	Univar. LC p-value*	Multivar. LC p-value*	
[11]	Chiba et al.	2010	Japan	Biopsy	IHC	GLUT-1	>30% membrane expression	SCC	25	28.0%	CCRT	14% vs. 67%	0.0185	28.5% vs. 73.4%	0.0003	0.007	yes
[16]	Koukourakis et al.	2001	Greece	Biopsy	IHC	HIF-1 α	Epithelial expression	SCC	37	51.0%	PDT	20% vs. 56%*	0.04	–	–	–	yes
						HIF-2 α	Epithelial expression	SCC	37	13.5%	PDT	0% vs. 42%	0.13	–	–	–	no
[22]	Ogawa et al.	2011	Japan	Biopsy	IHC	HIF1 α	>10% staining	SCC	25	42.7%	CCRT	15.4% vs. 84.6%	0.0027	42.7% vs. 72.5%	0.0322	0.178	no
[24]	Sohda et al.	2004	Japan	Biopsy	IHC	HIF-1 α	>10% cell staining	Mix	65	58%	CCRT	7.9% vs. 44.4%	0.009	–	–	–	yes
[28]	Winther et al.	2013	Denmark	Biopsy	Gene Clustering	15 hypoxia-assoc. genes	weighted hypoxia gene expression signature	SCC	56	31%	CCRT	33.3% vs. 52.4%	0.02	–	–	–	yes
[29]	Yue et al.	2012	China	Patient	PET scan	^{18}F-FETNIM	Ratio SUVmax to spleen SUVmean >1.3	SCC	28	82%	CCRT	42% vs. 100%	0.041 (SUVmax)	–	–	–	yes

low percentages of hypoxia-associated markers based on the reported threshold of hypoxia.

3. Results

3.1. Literature search

As presented in Fig. 1, a total of 419 records were initially identified in Web of Science (n = 182), PubMed (n = 215) using free-text search strings and in MEDLINE (n = 22) using MeSH-terms. After imposing language-restrictions and removing duplicate findings (n = 121), 244 full-text records remained. Further screening of records' title resulted in 85 potentially eligible studies by excluding articles that clearly stated terms did not fit the inclusion criteria (e.g., different tumor-types, reviews, meta-analyses, etc.). Next, abstracts of the remaining 85 articles were screened and based on the exclusion criteria, we excluded reviews (n = 4), papers that studied the molecular pathways of hypoxia (n = 7), and papers that did not evaluate esophageal cancer (n = 16), or treatment outcome (n = 13), or studied the effects of hypoxia (n = 14). Finally, the full-text content of 31 articles was assessed for eligibility and 11 studies were excluded for following reasons: studies had different study objective than our PICOS-based research question to assess esophageal tumor hypoxia (n = 6), the use of preclinical animal-based tumor models or esophageal cell lines (n = 4), and studies that did not allow the extraction of survival data or clinical effectiveness data (n = 1). Through citation tracking, additional papers (n = 2) were included that fitted the eligibility criteria. In total, 22 studies were included to be systematically reviewed [9–30].

3.2. Data extraction

Several studies confirm the presence of endogenous hypoxia-associated markers in esophageal cancer patients. The clinical impact of hypoxia in treatment outcome (i.e., OS, DFS, CR, and LC) is summarized in Tables 1 and 2, respectively. In both tables, clinical impact was defined by Kaplan-Meier analyses (log-rank test) and Cox proportional hazard model (uni- and multi-variate analyses).

In general, we found mainly endogenous tissue markers (HIF-1α, carbonic anhydrase IX and GLUT-1) with prognostic value and ability to predict treatment response in EC. We found only one study with non-invasive imaging including ^{18}F-FETNIM PET which correlated hypoxia to chemoradiotherapy response in EC.

3.2.1. Hypoxia-inducible factor (HIF)

Hypoxia-inducible factor (HIF) is the master protein in regulating the response of cells to changing oxygen levels and recognizes the hypoxia response element (HRE) on the untranslated region of over 150 genes involved in cell survival, tumor metabolism, proliferation, and angiogenesis [31,32]. HIF-1 exists as a heterodimer protein composed of constitutively expressed HIF-1β complexed with one of three subunits (HIF-1α, HIF-2α or HIF-3α). Synthesis of HIF-1α is regulated via O_2-independent mechanisms whereas degradation is primarily 0_2-dependent. Thus, HIF-1α upregulation could be a promising endogenous marker of hypoxia in EC. Interestingly, strong immunoreactivity for HIF-1α was presented more often in esophageal squamous cell carcinomas (ESCC) tumor tissue than in adenocarcinoma (AC) (p = 0.009) [25]. HIF-1α could be differently upregulated in ESCC than in AC. Together with molecular mutations and epigenetic alterations, the difference in outcome and treatment response of the two histologic subtypes could be explained [4]. Given the scarce data of HIF-1α in AC, no clear conclusion can be drawn regarding clinical outcome.

In a meta-analysis by Ping et al. [3], the prognostic significance of HIF-1α in ESCC has been investigated [3]. They reported that in univariate analyses HIF-1α overexpression was significantly associated with poor OS ($p < 0.001$, 10 studies), and DFS ($P = 0.013$, 2 studies). These findings are in accordance with most overlapping studies included in this review. However, Munipalle et al. [20] showed that HIF-1α overexpression was not correlated with OS in a European population ($p = 0.908$), contrary to the reported Japanese/Chinese population [20]. Presumably, this information was overlooked in the aforementioned meta-analysis because 11/12 studies included a Japanese or Chinese population (906/942 ESCC patients) and only 1 study was included with 36/942 ESCC patients originating from the UK. In multivariate analyses, opposing results have been presented as some studies indicated that HIF-1α overexpression is an independent prognostic factor for survival [21,27], while some studies report the contrary [17,18,30]. In the study by Zhang et al. [30], the prognostic power of HIF-1α overexpression could be lost by including metastatic/recurrent ESCC in the patient cohort [30]. Therefore, further clarification is needed in a large prospective study that includes both uni- and multivariate analyses to investigate differences in patient cohort, histological subtype, and pathologic origin (primary or metastatic EC).

In early stage esophageal cancer, HIF-1α expression in tumor tissue is associated with lower CR rates to local therapies such as photodynamic therapy (PDT) and concurrent chemoradiotherapy (CCRT) [16,22,24,29]. This suggests that low HIF-1α levels in EC may be a good indicator for early treatment response in otherwise treatment-resistant hypoxic tumors. The significant correlation between HIF-1α and CR has been confirmed by Ping et al. ($p = 0.001$, 4 studies) [3].

3.2.2. Carbonic anhydrase (CA IX)

Carbonic Anhydrase IX (CA IX) belongs to the family of zinc metalloenzymes with presence in normal stomach, intestinal and gall bladder tissue. It is involved in maintaining the cells pH-homeostasis by the reversible hydration of carbon dioxide into bicarbonate and hydrogen [33]. CA IX is over-expressed in hypoxic solid tumors through the HIF-1α activation cascade. Compared to HIF-1 α, CA IX is a stable and sustained marker of hypoxia with a half-life of 38 h [9,34]. In general, elevated membraneous CA IX was mainly found at the tumor center or at the border of tumors with expression rates being approximately 45–60% [9,12,26]. In 2008, Tanaka et al. reported that although hypoxia-induced CA IX expression correlated with more aggressive clinicopathological parameters and poor outcome, tumor related CA IX expression in ESCC was not an independent prognostic factor in multivariate survival analysis [26]. In contrast, Driessen et al. [12] showed that CA IX is a significant determinant in AC, and an independent prognostic factor for OS ($p = 0.017$) and DFS ($p = 0.041$) [12]. This was confirmed in a more recent study by Birner et al. [9] in an evenly-distributed patient cohort of ESCC and AC [9]. In a meta-analysis by van Kuijk et al. [33], EC-specific subgroup-analyses reported significant association between CA IX expression and both OS and DFS, respectively ($p < 0.001$) [33]. High CA IX expression was thus regarded as an adverse prognostic marker in EC. Furthermore, the expression of CA IX in tumor-surrounding stroma has also been significantly linked to shorter OS ($p = 0.013$) and DFS ($p = 0.007$) in a large cohort-study ($n = 155$ ESCC, $n = 206$ AC) [13]. It has been postulated that the difference in clinical behavior between ESCC and AC, could be related to a significant correlation between CA IX and HER-2 and/or a VEGF expression [9,12]. Nevertheless, these findings indicate the importance of this hypoxia-associated marker in disease progression and treatment resistance.

3.2.3. Other hypoxia-associated markers

The expression of glucose-transporter-1 (GLUT-1) is upregulated in hypoxic condition by HIF-1. In immunohistochemistry (IHC) analyses, GLUT-1 expression appeared to be a surrogate marker for hypoxia but also seemed to be prognostic factor for DFS and predictive for initial response to CCRT and LC [11].

Vascular endothelial growth factor (VEGF) is a transcriptional target for HIF and stimulates angiogenesis in EC [25]. Contradicting findings have been reported concerning the prognostic value of VEGF. In AC patients or in a mixed cohort, studies reported no association between VEGF expression and prognosis [12,25]. In ESCC, however, VEGF expression was regarded as an independent prognostic factor of OS [15,27].

3.2.4. Non-invasive imaging techniques

Non-invasive molecular imaging using positron-emission tomography (PET) has been shown to specifically detect hypoxic cell clusters in individual tumors using several 2-nitroimidazole derivatives [35–41]. Viable hypoxic cells are marked by 2-nitroimidazole derivatives through irreversible electron-reduction mechanisms involving nitroreductase enzymes such as cytochrome P450 reductase. Four clinically used, FDA approved hypoxia PET-tracers are presented in Table 3 [6,29,35,36,42–46].

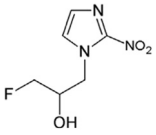
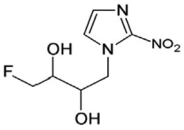
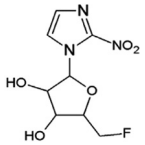
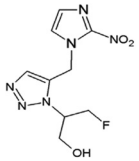
It has been shown that 18F-fluoromisonidazole (^{18}F-FMISO) allows visualization of hypoxic areas in a variety of tumors although data on EC remain scarce. In a study by Brink et al. [6], 33/38 patients with EC presented noticeable hypoxic volumes [6]. Standard uptake values (SUV) of ^{18}F-FMISO were shown to be significantly higher in AC ($n = 20$, SUVmean = 1.93 ± 0.43) than in ESCC ($n = 18$, SUVmean = 1.56 ± 0.25) ($P < 0.01$) [6]. However, the ability to visualize hypoxia differs in various cancer-types. For example, a significant correlation between ^{18}F-FMISO uptake and tumor markers from IHC (e.g., microvessel density, HIF-1alfa, VEGF, and GLUT-1) have been reported in head-and-neck cancer, whereas no correlation has been published in non-small-cell-lung cancer (NSCLC) [35,37].

In untreated ESCC, Yue et al. evaluated the spatiotemporal variability of hypoxia and assessed the ability to predict clinical response after CCRT using the PET-marker ^{18}F-fluoroerythronitroimidazole (^{18}F-FETNIM) [29]. In this study, ^{18}F-FETNIM presented pharmacokinetic advantages over ^{18}F-FMISO and SUVmax (^{18}F-FETNIM) was found to be predictive for clinical response to CCRT ($P = 0.041$). A higher baseline SUVmax (^{18}F-FETNIM) of 5.9 was found in non-responders, while complete or partial responders showed SUVmax (^{18}F-FETNIM) of 3.2 and 4.5, respectively.

Another promising PET-tracer able to visualize tumor hypoxia is ^{18}F-3-Fluoro-2-(4-((2-nitro-1H-imidazol-1-yl)methyl)-1H-1,2,3-triazol-1-yl)propan-1-ol (^{18}F-HX4) [36,47,48]. Klaassen et al. [44] first studied the feasibility and repeatability of ^{18}F-HX4 imaging in esophageal cancer [44]. Amount and location of elevated ^{18}F-HX4 uptake showed good repeatability in 19 EC patients (AD and SCC) suggesting that ^{18}F-HX4 PET could be a promising reliable tool to monitor tumor hypoxia in EC patients. Overall maximal tumor-to-background (TBRmax, mean ± SD) was found to be 1.87 ± 0.46 in EC, 4 h post-injection. ^{18}F-HX4 has proven to be clinically useful in the non-invasive detection of tumor hypoxia also in other tumor-types (e.g., head & neck and lung cancer) (Fig. 2) [37,38,40,49].

Although not yet assessed in EC, several other PET-tracer are known to visualize tumor hypoxia. For example, ^{18}F-FAZA has shown promising results in correlating hypoxia in head-and-neck cancer with outcome after CCRT [50]. Less popular clinical PET-tracers include non-nitroimidazole Cu-ATSM [Cu(II)-diacetyl-bis(N4-methylthiosemicarbazone)], ^{18}F-FETA, and ^{18}F-EF5. Although several studies have presented Cu-ATSM as a hypoxia marker for

Table 3
Overview of 2-nitroimidazole PET-tracers able to visualize tumor hypoxia in esophageal cancer. (*) missing data was substituted with known features in lung cancer. (**)Tumor to background ratio was defined as the ratio of SUVmax (tumor) over SUVmax (spleen).

	[18F]-FMISO	[18F]-FETNIM	[18F]-FAZA*	[18F]-HX4
Hypoxia PET-tracers				
Year of publication [44]	1987	1995	2002	2010
Stability	Metabolites in blood and urine	Fewer metabolite-formation than FMISO	Very few metabolite-formation (10–15%) [46]	Few metabolite-formation (18%) [47]
Clearance	Very slow Hepatobiliary	Slow Renal	Fast Renal	Very fast Renal
Optimal scantime	No plateau	2 h p.i. [29]	2 h p.i. [37]	3–4 h p.i. [45]
TBRmax (mean ± SD)	Not defined	2.41 ± 0.6* [37]	0.98 ± 0.19 [46]	1.87 ± 0.46 [45]
Hypoxic threshold	TBR > 1.2 [6]	TBR** > 1.3 [29]	TBR > 1.4 [82]	TBR > 1.0–1.4 [45]
Hydrophilicity (logP) [43]	0.4	−0.77	−0.4	−0.69

* NOT investigated in Esophageal cancer patients.
** SUVmax ratio Tumor-to-spleen.

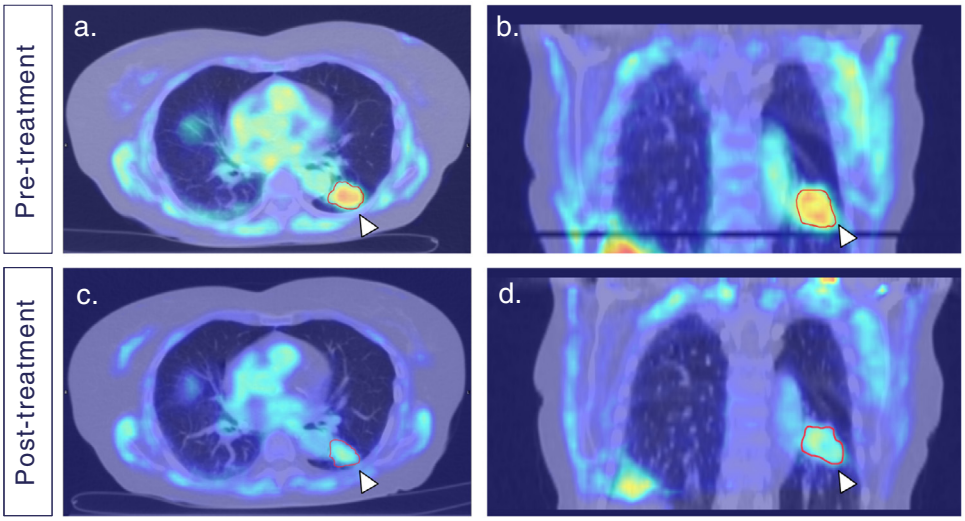


Fig. 2. Clinical ^{18}F-HX4 PET/CT imaging of hypoxic non-small cell lung cancer (4 h post-injection). The primary lung tumor (white triangle) is depicted in transversal (a,c) and coronal plane (b,d), measured before (a,b) and after hypoxia-modified chemoradiotherapy (c,d). SUV(^{18}F-HX4) ranged from 0.2 to 1.8.

radiation treatment outcome in rectal, lung, and head-and-neck cancer, cellular Cu-ATSM retention is affected by multiple mechanisms in addition to hypoxia [51,52]. Thus Cu-ATSM would not be a pure marker for hypoxia. Recently, ^{89}Zr-labeled cG250 monoclonal antibodies have been show to quantify and map CA IX expression in preclinical models and head-and-neck cancer using PET [53]. Directly labeling CA IX for hypoxia-related PET-tracers has also been investigated [54–56].

Ideal PET-tracers for hypoxia should be able to reach hypoxic cells in perfusion-limited microenvironments, have an oxygen-specific retention mechanism, and have a rapid and complete clearance of unbound radioactive tracer (i.e., hydrophilic) [42]. These properties ensure safe and optimal PET-imaging of tumor hypoxia. Unfortunately, none of the presented PET-tracers completely meet all these requirements (Table 3).

Another non-invasive technique that can detect hypoxia is magnetic resonance imaging (MRI) using exo- or endogenous contrast agents, and MR spectroscopy techniques such as electron paramagnetic resonance and hyperpolarized metabolic MRI [57]. Although MRI has not yet been used to investigate tumor hypoxia in EC, it shows promising results in other tumor types. In cervical cancer, for example, several studies have shown a correlation between tumor hypoxia and dynamic contrast enhanced (DCE-)MRI, using gadolinium bolus-injection, and demonstrated the ability to identify patients with hypoxia-related treatment resistance [58]. However, DCE-MRI estimates tumor perfusion and could therefore not assess the full extent of chronic tumor hypoxia. Similarly, blood-oxygen level dependent (BOLD)-MRI has been reported to be sensitive to tissue oxygenation by indirectly correlating deoxyhemoglobin-induced changes in MR signal to pO_2. In clinical trials, BOLD-MRI has been reported to map chronic hypoxic regions in prostate cancer by correlation with pimonidazole staining and evaluate hypoxia in breast cancer through correlation with CA IX expression ($r = 0.616$, $P < 0.001$) [59,60]. Although these MRI-techniques could be used as surrogate markers for tumor hypoxia, measurements are usually indirect and sensitive to image-related artifacts [61].

4. Discussion

In this study, we performed a systematic literature search, reviewed the various hypoxia-associated markers used in EC patients, and assessed the clinical impact of hypoxia in treatment

outcome (i.e., CR, LC, OS and DFS). Most included studies investigated invasively acquired hypoxia-associated markers (i.e., HIF and CA IX) in IHC analyses. Although all studies confirmed the presence of tumor hypoxia in esophageal cancer, the prognostic value was not consistent across all studies. Discrepancies might arise from methodology differences for hypoxia detection and quantification. In addition, diverging findings could arise from differences in tumor cell type (AC vs. SCC) or from population differences (Western vs. Far Eastern) in the study cohort. Nevertheless, HIF-1α overexpression could be regarded as a molecular biomarker for hypoxia-response and could be associated with treatment outcome and clinical response to CCRT and PDT in Asiatic patients with EC. In AC, CA IX levels in both tumor stroma and cell membrane are indicative for hypoxic status and prognostic for OS and DFS. However, we found contradicting results from multivariate survival analyses in studies with HIF-1α as well as with CA IX. These findings support the need to further elucidate the complex molecular mechanism of tumor hypoxia and construct more reliable prediction models. Because invasively acquired biomarkers report unreliable results and are lacking the ability to capture the full intricacies of tumor hypoxia and its heterogeneity, there is a need for robust and quantitative biomarkers to detect hypoxia or hypoxia-associated responses in EC and determine complete tumor oxygenation. Furthermore, clinical assessment of hypoxic status needs to be performed repeatedly (i.e., before and during (chemo)radiation treatment), since hypoxia is a dynamic process and reoxygenation could occur after irradiation [62,63]. Non-invasive imaging using radioactive PET-tracers shows great promise in repeatable and quantitative detection of hypoxic sub-regions in the entire tumor, although further validation of the clinical and prognostic value in EC is required.

Combining PET-imaging with MRI in a multimodal hybrid system (i.e., PET/MRI) might be the solution to identify potential new biomarkers and validate hypoxia-associated biomarkers in EC patients. Recently, Simoncic et al. [64] demonstrated a high correlation between ^{18}F-FMISO uptake parameters and DCE-MRI kinetic parameters in head-and-neck cancer patients (n = 6) [64]. However, the vascular data of dynamic PET and DCE-MRI was not exactly the same and the further development of simultaneous PET/MRI is encouraged to visualize hypoxic status. In addition to DCE- and BOLD-MRI, new techniques to assess tissue oxygenation are under development. Mapping oxygen by imaging lipids relaxation enhancement (MOBILE) detects variations in oxygenation based on MR relaxation rates of tissue lipids, instead of blood-oxygen related signal differences as seen in BOLD-MRI [65]. It has preclinically been confirmed that this novel technique is able to monitor changes in tumor oxygenation ($r = 0.51$, $p = 0.022$) and changes in lipid relaxation rates show moderate correlation with absolute pO_2 values ($r = 0.37$, $p = 0.027$) [66]. Another promising novel MR-technique is oxygen-enhanced (OE-)MRI, where tumor oxygenation is detected as oxygen-induced increase in MR signal that is generally larger than signal changes detected using BOLD-MRI or MOBILE. By letting subjects breath 100% oxygen, O_2-saturation in arterial blood plasma (Hb-bound and dissolved) will increase, resulting in an increase in tumor pO_2 and tissue oxygenation [67]. In a preliminary study, 10 patients with advanced abdominal/pelvic cancer underwent serial measurement of tumor relaxation rate while breathing medical air (21% oxygen) followed by 100% oxygen (OE-MRI). The resulting difference in MR signal was significant ($P < 0.005$), proving the ability of OE-MRI to indirectly detect changes in tumor oxygen levels [67,68]. When combining multiple MRI-techniques with simultaneous PET-based imaging (i.e., PET/MRI), complementary information in tumor perfusion, tissue oxygenation, metabolic activity, and oxygen consumption could be acquired. Similar to PET/CT, this multiparametric method could be used to validate novel hypoxia-associated biomarkers and may help elucidate the complex nature of chronic hypoxia [69]. Moreover, PET/MRI relies on highly sensitive PET-probes and highly specific anatomical and/or functional MR information. However, protocol standardization (i.e., execution and analyses) is needed to allow for reproducible results and validation method of hypoxia-detection in multiple clinics and tumor types.

Non-invasive imaging could be useful to monitor hypoxic status and estimate early clinical response during (chemo)radiation treatment. In non-responsive patients, treatment strategies could adapted to more hypoxia-guided therapies [70]. In radiotherapy, PET-based dose painting has been proposed to specifically deliver an escalated radiation dose or boost to hypoxic sub-volumes [71]. Such hypoxia-targeted radiotherapy could deliver an optimal dose distribution to radio-resistant regions. Currently, the survival probability of EC patients remains disappointing. Potentially, treatment outcome and patient survival could be improved by targeting hypoxia (i.e., increasing oxygen delivery, normalize tumor vasculature, or reduce oxygen consumption) or by implicating hypoxia-specific treatment strategies. However, pretreatment hypoxic status must first be assessed since large patient- and tumor-variability in oxygenation can exist. By selecting hypoxic patients before the start of treatment, a window-of-opportunity arises wherein attempts to reduce tumor hypoxia could be made. By first applying hypoxia-specific treatment strategies to overcome tumor hypoxia or eradicate hypoxic cells, conventional (chemo-) radiotherapy may become more effective and better treatment outcome can be achieved [47,70]. For example, preselected patients with hypoxic laryngeal cancer (i.e., high CA IX-fraction) had better LC and DFS when treated with accelerated radiotherapy with carbogen breathing and nicotinamide (ARCON) compared to accelerated radiotherapy (LC 97% vs. 71%, $p < 0.01$ and DFS 92% vs. 69%, $p = 0.06$) [72]. In contrast, a reversed scheme of radiation dose-painting has recently been proposed [73]. Here, hypoxic tumor regions were preclinically assessed using ^{18}F-HX4-PET/CT imaging and used for radiation treatment dose planning. Non-hypoxic regions (i.e., low ^{18}F-HX-4 uptake) received an escalated radiation dose, while hypoxic regions were targeted with hypoxia-activated prodrugs (HAP). Interestingly, this strategy was as effective as conventional radiotherapy plans but was able to reduce the mean overall tumor dose and hereby lowering normal tissue toxicity. Radiation dose was therefore used more efficiently.

It seems that hypoxia represents a 'Janus face' in tumor biology. On the one hand, it is associated with restrained proliferation and oxygen-deprived cell death, but on the other hand, it promotes adaptive processes leading to tumor aggressiveness, progression, and acquired resistance to treatment [74]. The high treatment failure rate seen in EC might therefore be due to a hypoxic microenvironment.

Molecular imaging could help individualizing hypoxia-specific treatment strategies in EC. Several approaches are available that focus on targeting HIF-1α and VEGF. YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole) suppresses esophageal tumor cell growth and inhibit cellular migration activities [75]. Similarly, radiosensitivity could be enhanced by downregulating VEGF and HIF-1α protein levels. Drugs such as Ginsenoside Rg3, Fenofibrate, and Berberine have been associated with anti-tumor and anti-angiogenesis activities by promoting radiosensitivity of human hypoxic EC cell lines [76–78]. By targeting CA IX expression, therapeutic benefit could be improved when combining conventional treatment with cytotoxic agents such as CA IX-directed ligands or antibodies [79].

Another hypoxia-specific treatment strategy is the use of hypoxia-activated prodrugs (HAP) that become activated by enzymatic reduction under hypoxic conditions to release cytotoxic

effectors (“warheads”). For example, evofosfamide (TH-302) is a HAP that upon activation in severely hypoxic regions induces DNA damage but also diffuses to the surrounding, better oxygenated, cells and creating cytotoxic bystander effects. TH-302 has demonstrated enhanced anti-tumor effects in combination with (chemo)radiotherapy, although levels of toxicity were also elevated [47,80]. In addition, significant clinical benefit has yet to be reported for treatment strategies involving currently-available HAP (i.e., monotherapy or combined with chemoradiotherapy) [81]. We acknowledge the potential therapeutic effect of additional anti-hypoxia treatment, but also the importance to limit unnecessary toxicity by selecting patients who will benefit from these modifications. Extensive clinical testing of TH-302 in combination with CCRT is therefore advised in pre-selected hypoxic patients using for example HX4-PET imaging [70,80].

Although this systematic review adheres to the PRISMA statement, it holds a few limitations [8]. Several clinicopathological factors such as age, clinical stage, lymph node invasion, and location (i.e., proximal or distal EC) were not investigated, but could explain the different expression rate of hypoxia-associated biomarkers in esophageal cancer. Furthermore, interactions between these factors and treatment differences (e.g., radiation dose, fractions, and chemo regimens) were not described. Beside hypoxia, reactive oxygen species, genetic alterations and inflammation may also be involved in the stimulation of hypoxia-associated molecular responses. Finally, we did not assess the effect of methodological differences across included studies (e.g., IHC staining procedures, antibody supplier, slice thickness, and threshold for hypoxic status). We assumed that reliable, standardized protocols were applied correctly for optimal detection of hypoxia-associated markers. Although presumed to be inconsequential, the possibility of impure comparison emphasizes the need for protocol standardization.

5. Conclusion

Evaluation of tumor micro-environmental conditions, such as intratumoral hypoxia, is important to predict treatment outcome and efficacy. Until now, the predictive value of hypoxia-associated biomarkers in esophageal cancer is controversially discussed. Although there is increasing clinical evidence that hypoxia-associated responses can be detected, the perfect biomarker for tumor hypoxia in EC has not yet been established. However, PET-based hypoxia imaging shows great potential in evaluating hypoxic tumor status non-invasively. Knowledge of the presence and dynamics of hypoxia in different esophageal cancer patients (ESCC vs. AC) is important to exploit and validate novel therapeutic strategies directed against tumor hypoxia. The window-of-opportunity trial concept paves the way for optimal hypoxia diagnosis and individualized hypoxia-guided treatment to improve radiotherapy response in EC patients. For personalized cancer medicine, simple, safe, and efficient methods are needed to determine tumor oxygenation in EC and help select patients with hypoxic tumors. Presumably, the combination of multiple, minimally invasive molecular markers is needed to fully evaluate the hypoxic status in cancer patients.

Disclosure of interest

The authors report no conflict of interest.

Funding

L.D. was funded by Worldwide Cancer Research (Worldwide Cancer Research), formerly known as AICR [Grant #15-0345].

Appendix A

A1. Systematic search protocol

Research question

How does hypoxia affect treatment efficacy and outcome in patients with esophageal cancer?

Search strategy

Web of Science: 182 hits. **TOPIC:** (hypoxia) *OR* **TOPIC:** (hypox*) **AND TOPIC:** TS = (cancer) OR TS = (tumor) OR TS = (tumor) OR TS = (carcinoma) OR TS = (neoplasm) OR TS = (oncology) OR TS = (lymph node) **AND TOPIC:** (esophag*) *OR* (oesophag*) *OR* (esophageal cancer) *OR* (oesophageal cancer) *OR* (esophageal carcinoma) *OR* (oesophageal carcinoma) *OR* (esophageal tumor) *OR* (oesophageal tumor) **AND TOPIC:** TS = (radioresistance) OR TS = (prognosis) OR TS = (treatment outcome) OR TS = (tumor aggressiveness) OR TS = (tumor spread) OR TS = (malignant progression) OR TS = (metastasis) OR TS = (clinical outcome) OR TS = (response prediction) OR TS = (pathological free survival) OR TS = (non-responders) OR TS = (pathological response) OR TS = (treatment resistance) OR TS = (therapy resistance) OR TS = (treatment efficacy).

MEDLINE (MeSH): 22 hits. Search (((((((hypoxia[MeSH Terms]) **AND** ((((cancer[MeSH Terms]) OR oncology[MeSH Terms]) OR tumor[MeSH Terms]) OR tumor[MeSH Terms])) **AND** ((((((radiation therapy[MeSH Terms]) OR treatment outcome[MeSH Terms]) OR prognosis[MeSH Terms]) OR adjuvant chemotherapy[MeSH Terms]) OR immunotherapy[MeSH Terms]) OR treatment resistant[MeSH Terms])) **AND** (((((esophageal cancer[MeSH Terms]) OR **oesophag***[MeSH Terms]) OR esophag*[MeSH Terms]) OR lymph node[MeSH Terms]) or mediastin* [MeSH Terms]))))).

PubMed (Free text): 215 hits. ((((((((hypoxia) OR hypox*) OR hypoxia-induced factor)) **AND** ((((((((((esophageal cancer) OR esophag*) OR oesophageal cancer) OR mediastinum) OR lymphadenopathy) OR oesophageal carcinoma) OR esophageal tumor) OR oesophageal tumor) OR esophageal carcinoma) OR lymph node) **AND** ((((((((((((((radioresistance) OR prognosis) OR treatment outcome) OR tumor aggressiveness) OR tumor spread) OR malignant progression) OR metastasis) OR clinical outcome) OR response prediction) OR pathological free survival) OR non-responders) OR pathological response) OR treatment resistance) OR therapy resistance) OR treatment efficacy)).

Inclusion criteria

P = PARTICIPANTS OR PATIENTS Species: human.

Age: adults (minimal age >18 yr.).

Sex: no restriction.

Condition: esophageal cancer.

Specimen: patients or biopsy-acquired tissue samples or surgical tissue samples.

Tumor type: Squamous cell carcinoma (SCC) and adenocarcinoma (AC).

Stage: no restriction.

I = INDEX TEST. All clinical tests able to measure hypoxia-related markers.

C = COMPARATIVE TEST. not relevant.

O = OUTCOME. Overall survival (OS), Disease-free survival (DSF).

S = STUDY DESIGN. Study design: original diagnostic experiments.

Study type: full-text content available, no studies aiming to elucidate molecular mechanisms of hypoxia.

Language: English.

Publication year: no restriction.

Exclusion criteria

Based on title & abstract.

Based on full text.

1. Irrelevant study, not meeting PICOS-characteristics.
2. preclinical studies using animal-based tumor models or esophageal cell lines.
3. Reviews, letters to the editor, comments, supplements, conference abstracts, reports, essays, symposiums, guidelines.
4. Overlapping data-sets.
5. Survival analyses were not presented in publication.
6. Presented survival analyses did not allow correlation between expression rate of hypoxia-associated markers and treatment outcome analyses.

Data extraction

Elements that were extracted comprised of:

Patient characteristics: number of subjects included in the study, country of origin, mean age of the patient population, tumor cell type, specimen type.

Index test characteristics: method of quantifying hypoxia, marker type, definition of hypoxia, percentage of hypoxic elements, selected treatment strategy.

Outcome parameters: statistical analysis (uni- or multivariate), overall survival, disease free survival, complete response, local control, p-values indicating statistical difference in treatment outcome between high and low percentages of hypoxia-associated markers.

Study characteristics: First author, publication year.

Finally, each article was given a unique identification number.

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